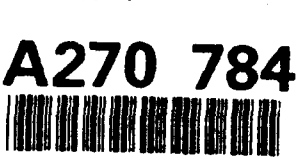

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The second method of evaluation—degree of lesion pathology—was determined by observing the percent incidence (percentage of exposure sites with positive response) of several parameters, including (in order of increasing dose dependence) intracellular edema, basal cell necrosis, pustular epidermitis, microblister formation, and follicular necrosis. All markers exhibited a linear dose-response curve with parallel slopes. The more extensive cellular damage occurred at longer exposure times. We have observed in the hairless guinea pig microblister formation at the dermal-epidermal junction similar to that reported in other animal models including humans. The linear dose-response curve for microblister formation correlated highly with available historical data for fluid-filled blisters in humans under similar exposure conditions

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Characterization of the Sulfur Mustard Vapor Induced Cutaneous Lesions on Hairless Guinea Pigs

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Summary: The identification of antivesicant compounds has been hindered by the lack of a suitable in vivo model. Our laboratory has been evaluating the hairless guinea pig as a useful animal model to mimic the human cutaneous response to sulfur mustard (HD) exposure. The characterization of two cutaneous responses to HD, quantification of erythema using a reflectance color meter, and pathology by light microscopy, is described in this manuscript. Fifty-two hairless guinea pigs were exposed to saturated HD vapor using a vapor cup technique. The dose response of HD vapor was established by varying the duration (2–11 min) of skin exposure to an HD vapor concentration of ~0.77 g/m^3 under occluded caps. The degree of erythema was determined by measuring the increase in the reflectance meter's **a*** (relative parameter measuring the degree of redness) response for each exposure site at 4, 5, 6, and 24-h postexposure. A linear increase in erythema was observed between the 2- and 4-min HD exposure doses for each postHD observation. The increase in erythema reached a maximum with the 5-min HD exposure dose. HD exposures between 5 and 11 min produced the same degree of erythema. The second method of evaluation—degree of lesion pathology—was determined by observing the percent incidence (percentage of exposure sites with positive response) of several parameters, including (in order of increasing dose dependence) intracellular edema, basal cell necrosis, pustular epidermitis, microblister formation, and follicular necrosis. All markers exhibited a linear dose-response curve with parallel slopes. The more extensive cellular damage occurred at longer exposure times. We have observed in the hairless guinea pig microblister formation at the dermal-epidermal junction similar to that reported in other animal models including humans. The linear dose-response curve for microblister formation correlated highly with available historical data for fluid-filled blisters in humans under similar exposure conditions. **Key Words:** Sulfur mustard—Hairless guinea pig—Erythema—Reflectance color meter—Histopathology—Vesicant animal model.

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The chemical weapon mustard gas, 2,2'-dichlorodiethyl sulfide (HD), has remained a serious threat to both civilian and military populations since World War I. Identification of compounds that are effective in the treatment and prevention of injury caused by vesicants, such as HD, is hampered by the lack of in vivo models. A model is needed for the quantitative and rapid assessment of the efficacy of prophylactic and therapeutic drugs. A good animal model should have vesicant pathophysiology comparable to that of humans, a wide range of dose-related responses that are readily measurable and reproducible, and no requirement for specialized care or handling before and after agent dosing. Initial studies by Marlow (1) demonstrated that in the hairless guinea pig exposure to neat liquid HD produces damage that is not dose-dependent and much too severe for the purpose of evaluating potential antivesicant compounds. Additional work was done by Mershon (2), who adapted a vapor cup exposure technique for applying HD vapor to the skin of hairless guinea pigs. This vapor exposure technique produces more uniform and reproducible lesions on the hairless guinea pig than does the liquid exposure method. These vapor cup studies have demonstrated the suitability and versatility of the hairless guinea pig as a vesicant model. Topical exposure of hairless guinea pigs to HD vapor produces highly reproducible lesions that vary in severity in a dose-dependent fashion. A 4-min HD vapor exposure is moderately damaging. Visual lesion evaluation consistently produces a median Draize erythema score of 2–3 and an edema score of 1–2. Epidermal injury is characterized histologically (1) by minimal to mild intra- and extracellular edema, rare isolated epithelial necrosis, and the occasional presence of neutrophils. Dermal involvement consists of minimal to mild edema and congestion with a consistent increase in numbers of inflammatory cells (primarily neutrophils). Microblister formation is rare with a 4-min exposure. The 8-min exposures are severely damaging, resulting in median Draize scores for both erythema and edema of 3–4. An 8-min HD exposure results in an increase in severity of the histological changes noted with a 4-min exposure. Additionally, loss of cell-to-basal lamina and cell-to-cell adherence results in a high incidence of microblister formation. The microscopic lesions observed in these animals were comparable to those reported by Marlow (1) for the hairless guinea pig and by Mitcheltree (3) for swine exposed to liquid HD. Unlike liquid HD contact exposure wherein microblisters are observed primarily on the periphery of areas of severe coagulation necrosis, exposure to HD vapor produces microblisters or clefts involving the entire exposure site. Although frank, fluid-filled blisters are not observed, histological separation between dermis and epidermis is highly consistent with blister formation. The most severe lesions grossly resemble a blister without fluid at 24 h postexposure. In some animals, the epidermis peels from the entire exposure site during collection of skin specimens for pathology.

This preliminary work demonstrates that the hairless guinea pig offers several advantages over haired guinea pigs as a cutaneous vesicant animal model. These advantages include (a) greater sensitivity to HD, (b) simplified application of both liquid and vapor HD, (c) ease of visualizing and evaluating developing skin lesions, and (d) a higher incidence of microblister formation.

There have been many recent reports (4–10) indicating that a reflectance color meter can be used for the quantitative evaluation of human skin erythema. We have recently reported (11) that a reflectance color meter (Chroma Meter Model CR-200,

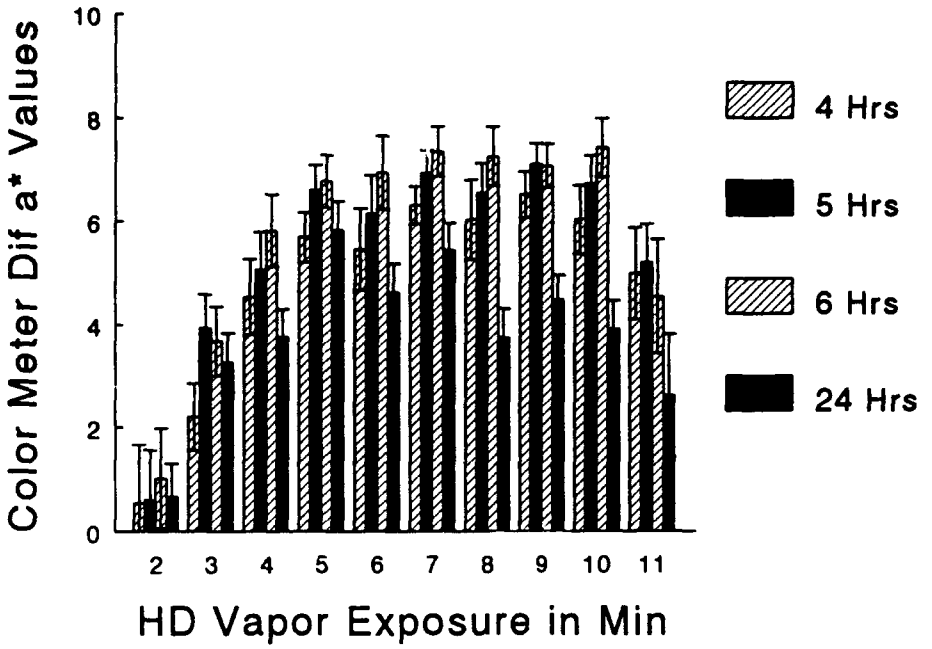

FIG. 1. Dose-response curve for erythema in hairless guinea pig following saturated HD-vapor cutaneous exposure. Δ**a*** chromaticity values reflect the increase in redness observed on the skin surface. Data for the 3–10-min exposures were collected on 24 hairless guinea pigs with two sites per animal yielding an n of 48. Data for the 2–11-min exposures were collected on four animals with four sites per animal yielding an n of 16. Error bars represent the 95% CIs.

Minolta Corporation, Ramsey, NJ, U.S.A.) is capable of providing a reproducible and quantitative assessment of the degree of erythema produced on the skin of the hairless guinea pig following exposure to HD vapor. The color meter's **a*** chromaticity coordinate closely parallels the visual Draize (12,13) evaluation scores. The **a*** reading provided by the color meter is one of three coordinates (**L***, **a***, and **b***) of a three-dimensional color system recommended by Commission Internationale de l'Eclairage (CIE) (14). The **a*** coordinate represents the relative chromaticity between red (+60) and green (−60) and has been observed to closely parallel the response to red color observed by humans (11). A complete discussion on CIE color systems may be found in the book by Hunt (15).

Before using the hairless guinea pig model to evaluate potential antivesicant compounds it was necessary to define and validate end-points that were related to the cutaneous response observed in humans. To meet this need we now report two end-points in the hairless guinea pig that are useful in the evaluation of antivesicant compounds and provide detailed dose-response curves for the cutaneous HD vapor exposure to the backs of these animals. The erythema response was determined using a reflectance color meter and the tissue damage was determined by histopathological evaluation.

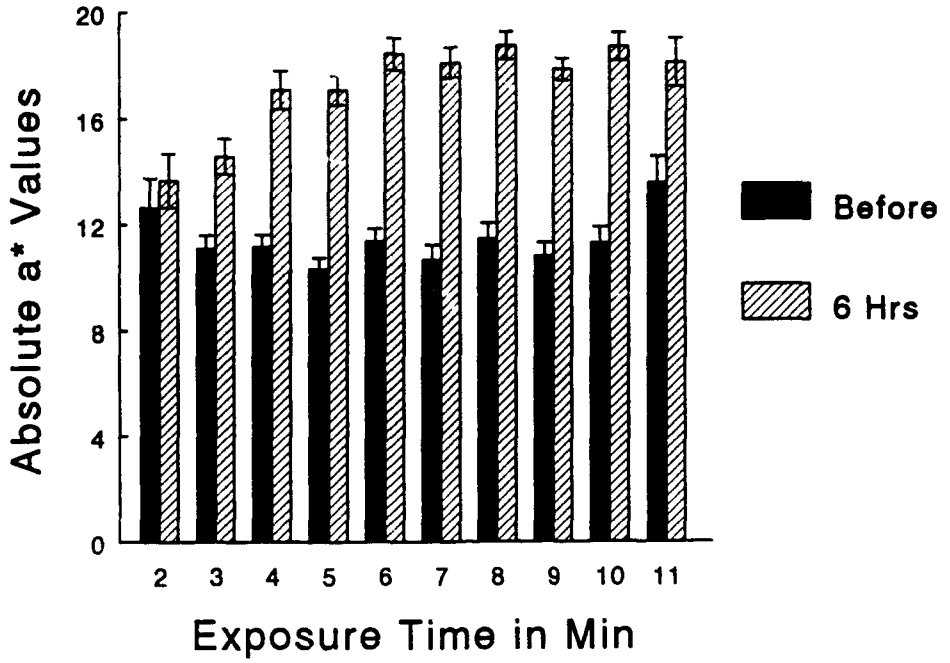

FIG. 2. Absolute reflectance color meter readings for 2–11-min HD-vapor cutaneous exposure to hairless guinea pig observed before exposure and at 6 h HD exposure. **a*** chromaticity values represent the degree of redness observed on the skin surface. Data for the 3–10-min exposures were collected on 24 hairless guinea pigs with two sites per animal yielding an n of 48. Data for the 2–11-min exposures were collected on four animals with four sites per animal yielding an n of 16. Error bars represent the 95% CIs.

MATERIALS AND METHODS

Animals

A total of 52 male Crl:IAF/HA(hr/hr)BR Vaf/PlusU/U airless guinea pigs (*Cavia porcellus*), 260–560 g, were used in these experiments. Guinea pigs were quarantined upon arrival and screened for evidence of disease before being released from quarantine. They were maintained under an American Association for Accreditation of Laboratory Animal Care accredited animal care and use program in polycarbonate plastic cages (Lab Products, Maywood, NJ, U.S.A.) on corn cob bedding (Bed-o-cobs; Industrial Products Div, Maumee, OH, U.S.A.) and provided commercial guinea pig ration (Zeigler Bros., Gardners, PA, U.S.A.) and tap water ad libitum. Animal holding rooms were maintained at 70 ± 2°F with 50 ± 10% relative humidity using at least 10 complete changes per hour of 100% conditioned fresh air. Guinea pigs were on a 12 h light/dark full spectrum lighting cycle with no twilight. Animals were killed by an inhalation overdose of halothane at the end of the experimental period.

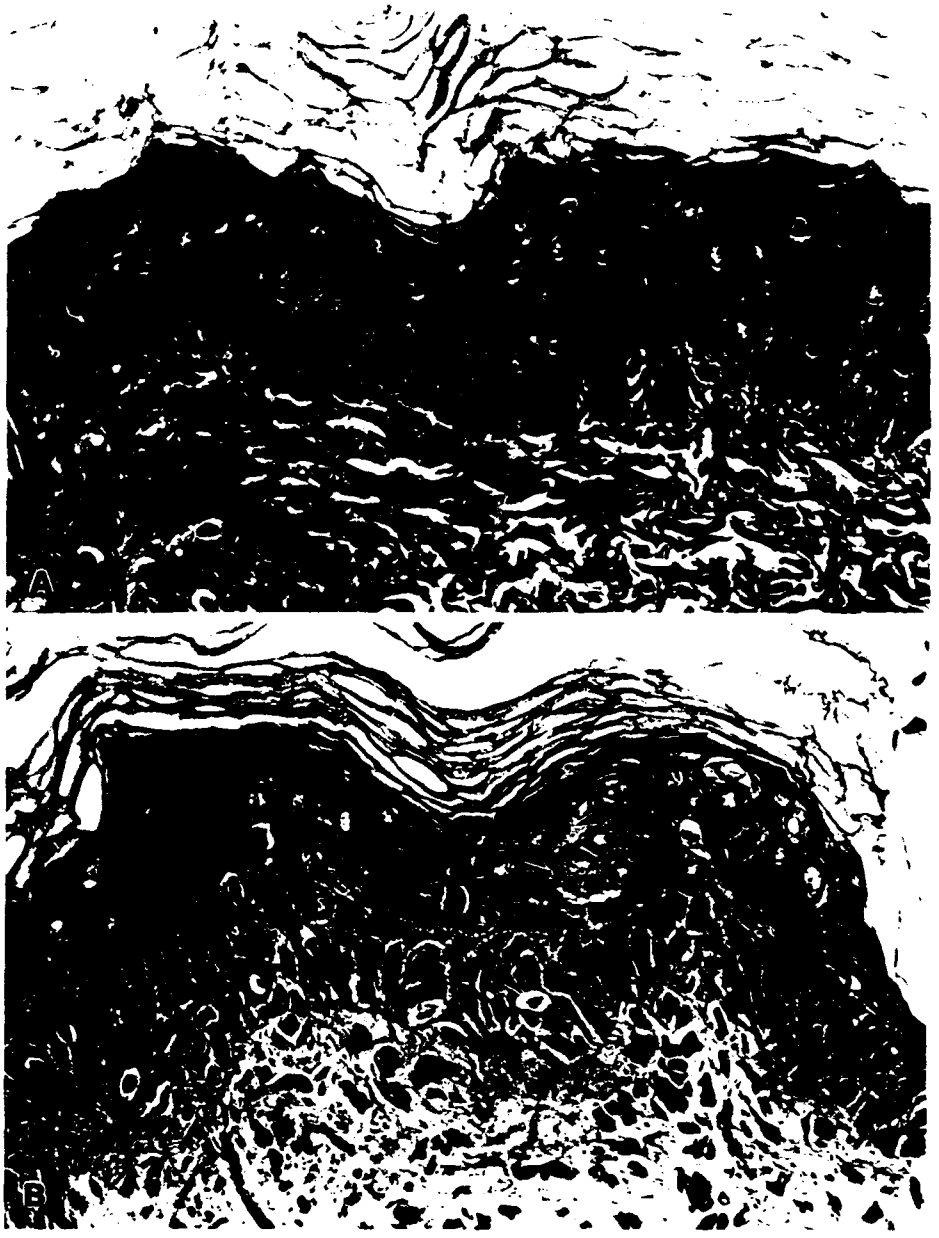

FIG. 3. Skin sections of hairless guinea pigs exposed to various doses of HD vapor. **A:** Skin taken from the dorsal lumbar area of an unexposed hairless guinea pig (H & E. ×200). **B:** Section of skin 24 h after being exposed to saturated HD vapor for 3 min. Early microscopic changes consisted of disruption of the basal cell layer and small multifocal areas of intracellular edema (ballooning degeneration, arrows) and intercellular edema involving several layers of the epidermis (H & E. ×200). **C:** Section of skin 24 h after being exposed to saturated HD vapor for 5 min. The intracellular edema (arrows) has become more pronounced. Several cells have

become necrotic (pyknotic nuclei) and small numbers of neutrophils have infiltrated the epidermis. Necrosis of the superficial follicular epithelium (fe) is also present (H & E. ×200). **D:** Section of skin 24 h after being exposed to saturated HD vapor for 7 min. Ballooning cells (arrows) with pyknotic nuclei coalesce and begin to form a pre-microblister (H & E. ×200). **E** (p. 248): Section of skin 24 h after being exposed to saturated HD vapor for 9 min. Separation and evaluation of epidermis from dermis, forming a large microblister (MB), is partially filled with fibrin (f), necrotic debris, and occasional neutrophils and macrophages. Acantholysis (arrows) (H & E. ×200).

FIG. 3—*Continued.*

Experimental Design

Fifty-two animals were randomly divided into three groups. Groups A and B each had a total of 24 animals, subdivided into six sets of four animals each. Group C had four animals divided into two sets of two animals each. Group A animals were exposed to HD vapor for 3, 5, 7, and 9 min on four contralateral pairs of skin sites. Group B animals were exposed to HD vapor for 4, 6, 8, and 10 min on four contralateral pairs of skin sites. Contralateral pairs of exposure sites were successively rotated on animals to preclude anterior/posterior sensitivity biases. On each exposure day, one set of four animals was exposed from groups A and B. Group C animals were all exposed on the same day to HD vapor for 2 and 11 min on four contralateral pairs of skin sites. In one set of two animals, the four 2-min exposures were on the right side while the four 11-min exposures were on the left side. Of the second set of two animals, the positions of the 2- and 11-min exposures were reversed.

Technique

The back of each animal was cleaned with an alcohol pad (alcohol prep antiseptic, NDC 19154-1245-3, The Clinipad Corp., Guilford, CT, U.S.A.) 18–22 h before exposure to remove soil and debris. The HD vapor exposure technique has been previously described (11). Briefly, hairless guinea pigs were randomly assigned and anesthetized with an intramuscular combination of 30 mg/kg ketamine HCl (Vetalar, 100 mg/ml, Parke-Davis, Division of Warner-Lambert Co., Morris Plains, NJ, U.S.A.)

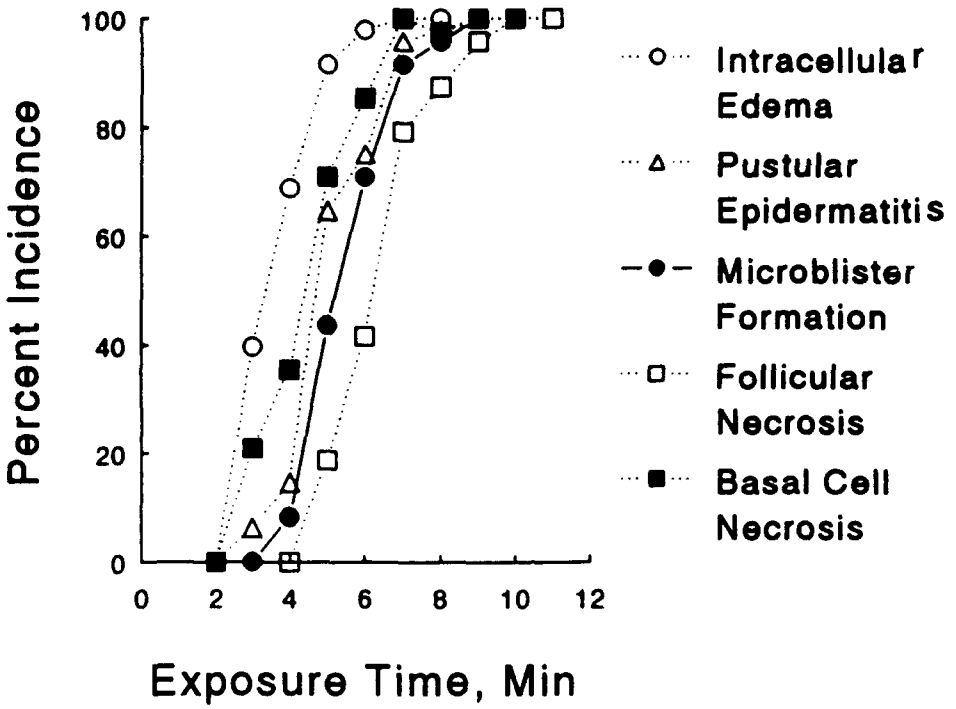

FIG. 4. Dose-response curve for histopathology parameters in hairless guinea pigs following saturated HD-vapor cutaneous exposure. Percent incidence is determined by whether or not the indicated parameter was observed by light microscopic examination. Data for the 3–10-min exposures were collected on 24 hairless guinea pigs with two sites per animal yielding an n of 48. Data for the 2- and 11-min exposures were collected on four animals with four sites per animal yielding an n of 16. (· · ○ · ·) intracellular edema, (· · ■ · ·) basal cell necrosis, (· · △ · ·) pustular epidermitis, (— ● —) microblister formation, and (· · □ · ·) follicular necrosis.

and 6 mg/kg xylazine (Rompun, 20 mg/ml, Mobay Corp., Animal Health Division, Shawnee, KS, U.S.A.) and placed in sternal recumbency. Animals were draped to expose only the back area used in the experiment. The cranial and caudal boundaries of each exposure site were marked with an indelible pen to localize each site in the absence of visible changes. Exposure sites were successively rotated on animals to preclude anterior/posterior sensitivity biases. The HD vapor dose, C^t, was established by varying the duration, t, of skin exposure to the concentration, C, of HD vapor generated under polyethylene caps 14 mm wide and 5 mm deep (No. P799C, Columbia Diagnostics, Inc, Springfield, VA, U.S.A.). Caps were fitted with 14-mm discs of Whatman No. 2 filter paper, fixed 5 mm above the cap rim. The filter paper in each inverted cap was wetted with 10 μl of neat HD. Previous studies by Mershon (2) had established that this volume of HD was sufficient to wet the filter paper without run-off. Loaded caps were stored with the rims on glass microscope slides for at least 5 min prior to transfer onto animals to establish an HD vapor pressure. Vapor caps

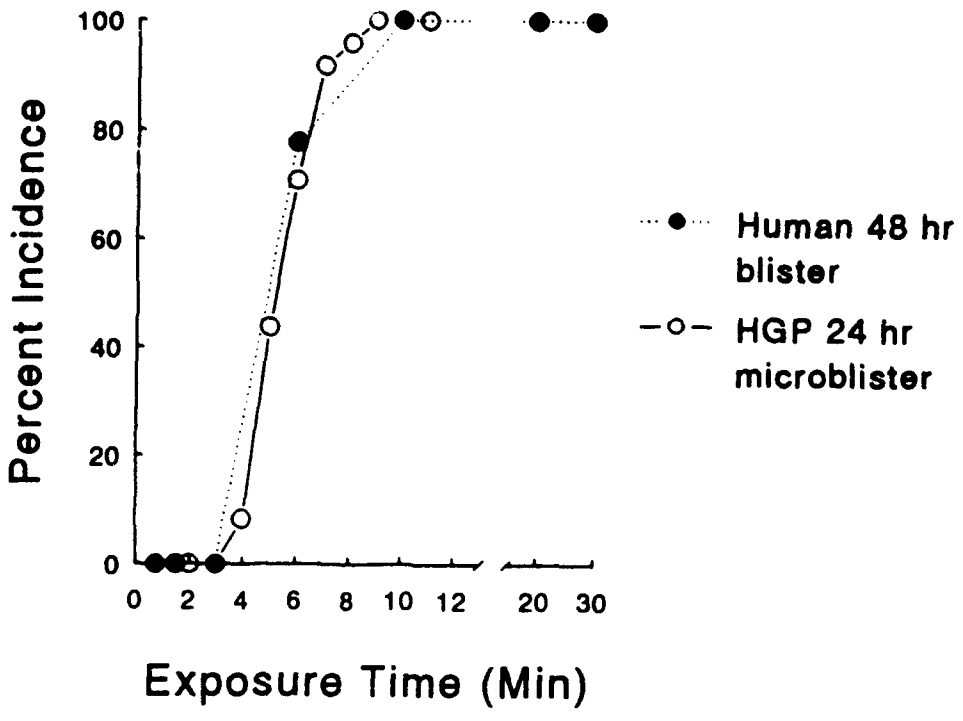

FIG. 5. Dose-response curves comparing the percent incidence of microblister formation in hairless guinea pig to the available historical data base for blister formation in humans following cutaneous HD-vapor exposure under similar conditions. (· · ● · ·) human 48-h fluid-filled blister and (· · ○ · ·) hairless guinea pig 24-h microblister.

were held to the skin by double-sided tape assemblies. Although the HD vapor concentration under the caps was not determined, it was estimated from the equilibrium vapor pressure (16) of 0.090 mm Hg at 30°C. This corresponded to a vapor concentration of 770 mg/m^3 HD. After removal of caps and tape, animals were placed in individual cages between observations. Animals remained anesthetized for a period of ~1.5–2 h. After anesthesia, animals did not display any signs of distress or discomfort during the 24-h observation period.

Exposure sites on the guinea pigs were evaluated for erythema before vapor exposure (control readings) and at various time intervals after exposure using a reflectance color meter (Minolta). At 24-h postexposure, tissue damage was evaluated by light microscopy. Briefly, the Minolta Chroma Meter model CR-200, previously described (11), includes a small hand-held measuring head that is easy to position on the animal for readings. The area evaluated by the head was a circle 8 mm in diameter. Four replicate color meter readings were recorded for each exposure site as pretest or control readings prior to induction of anesthesia. Four replicate readings for each site were recorded again at 4, 5, , and 24 h postHD exposure. The Δa^* color meter values represent the increase in erythema associated with each exposure site at the indicated observation times. The increase in erythema was determined from the

calculated difference between control readings and readings at each postHD exposure observation time. Ninety-five percent confidence interval (CI) error bars were calculated (17) by the formula $\mu = \bar{x} \pm t(s/n^{1/2})$ where μ = population mean, $\bar{x}$ = sample mean, t = t-value distribution, s = the standard deviation (unbiased n − 1 formula), and n = the total number of observations (no. of animals times no. of sites per animal). For the HD exposure times of 3–10 min, 24 animals with two sites each gave an n of 48. For the HD exposure times of 2 and 11 min, four animals with four sites each gave an n of 16.

After the 24-h color meter readings, animals were immediately killed, after which the skin covering the backs of the animals was removed and 8-mm skin punches were taken from each of the exposure sites. The skin punches incorporated the entire central portion of the exposed area. The skin punches were immersed in 10% neutral buffered formalin for a minimum of 24 h after which they were bisected. Both halves were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (H & E). The central straight cut surface was the focus of histologic examination. From each site, two sections of each half (total of four sections) were examined for pathologic changes. On microscopic examination, particular attention was centered on the basal cell layer. A microblister was identified only if (a) a continuous span of basal cells (minimum 4–6) had undergone necrosis and dissolution (cytolysis) *and* (b) no visible membranous attachments to the underlying region of the basal lamina were observed in any one of the four sections.

RESULTS AND DISCUSSION

Erythema Evaluation

The degree of erythema was evaluated using a reflectance color meter. As was observed in our previous experiments, the maximum erythema was reached in 5–6 h postHD exposure, with a significant decrease observed at the 24-h observation (Fig. 1). At all exposure times, except for 2 and 11 min, there was a significant increase ($p < 0.05$) in the erythema between 4 and 6 h postHD observations but not between 5 and 6 hr readings. A dose-dependent increase in erythema was observed between the 2- and 4-min HD exposure times for all observations. The increase in erythema began to level off after 4 min of HD exposure and reached a maximum for the 5-min HD exposure. HD exposures between 5 and 10 min produced the same degree of erythema. The 11-min HD exposure time showed a significant decrease in the Δ **a*** readings at all postHD exposure observation times. The observed decrease at an 11-min exposure was not consistent with other experimental data (unpublished results from our laboratory). After reviewing the data, it was determined that the reason such a small increase in Δ **a*** was observed was that many of the exposure sites on the four animals used in this experiment were very red to begin with. A comparison (Fig. 2) of absolute **a*** values for the preHD and 6 h postHD data over the exposure times of 2–11 min illustrated that the preHD readings for a 2- and 11-min exposure time are significantly higher than the **a*** values for the 3–10-min exposures. The absolute **a*** values for the 11-min exposure, however, were not significantly less than the values for the 5–10-min exposures. Similar trends were observed for the other observation times. This analysis suggested that the 11-min **a*** difference values were less

than expected due to the higher than average values before readings. Hairless guinea pigs apparently reach a maximum erythema that corresponds to an absolute mean **a*** reading of ~19. Even though the absolute **a*** readings were also high for the preHD 2-min exposure times, the difference values were probably not suppressed since only a small increase was observed and the erythema **a*** readings were well below the maximum possible response. These data demonstrated the importance of screening animals for redness before using them in experiments.

The dose-response curve of the 24-h observation was observed to increase in a dose-dependent way for low HD exposures (2–5 min). At exposure times > 5 min the degree of erythema was observed to be less than the maximum. This was consistent with the extent of tissue damage discussed in the next section. At low HD doses, only minor tissue damage was observed and epidermal cells remained viable. By the 24-h observation, the erythematous stage of lesion development was near completion. As the HD dose was increased, however, progressively more tissue damage occurred and, by 24 h, the exposure sites became progressively more necrotic and displayed a pale yellow color. This trend was consistent with the decrease in observed **a*** readings. Other noninvasive lesion evaluation methods, such as laser Doppler flowmetry and skin elasticity, are not yet available in our laboratory. We plan to acquire capability for these techniques in the future and will assess their utility in the evaluation of HD-induced cutaneous lesions.

Histopathology Evaluation

All skin specimens were taken after death at 24 h following HD vapor exposure. No significant pathological changes were observed following the 2-min exposure when compared to control (Fig. 3A). A mild increase in the numbers of neutrophils, however, was noted diffusely throughout the dermis. The earliest microscopic lesions were observed in ~40% of the sites following a 3-min exposure and consisted of small multifocal areas of intracellular edema (ballooning degeneration) involving both superficial and deep layers of the epidermis (Fig. 3B). This change was observed in all sites at exposure times of ≥7 min.

Basal cells became extremely swollen and sometimes ruptured (basal cell necrosis), beginning as early as a 3-min exposure and increasing in severity, distribution, and frequency with increasing time of exposure (Fig. 3C). As increasing numbers of cells ruptured, adjacent spaces coalesced to form small clefts and microvesicles (microblisters) (Fig. 3D). Microblisters were initially observed in a few sites following a 3-min exposure and reached 100% occurrence at a 9-min exposure (Fig. 3E). Almost paralleling the incidence of basal cell necrosis was the influx of neutrophils into the affected epidermis (pustular epidermitis). These began to appear in some sections at the 4-min exposure and increased in frequency and distribution until peaking at 100% with 9 min of exposure (Fig. 3E).

Necrosis of epithelial cells lining the hair follicles (follicular epithelial necrosis) below the level of the dermal-epidermal junction first became evident in a few sites after a 5-min exposure (Fig. 3C). It was never present unless all of the preceding changes were also present and involved all sites following 10 min of exposure. Changes noted in all sections, regardless of time exposure, consisted of widening

spaces between dermal collagen bundles (dermal edema) and congestion of small vessels. These cellular markers all exhibited a linear dose-response curve with parallel slopes (Fig. 4). The more severe cellular damage occurred at longer exposure times. The HD vapor-induced skin pathology that we have observed in this study is similar to that observed in other animal models, including humans. A detailed review of the literature is available in a recent book by Papirmeister (18).

Of the histopathology parameters that we have evaluated following an HD vapor challenge in the hairless guinea pig, microblister formation best approximates the macroscopic fluid-filled blisters observed in humans. Figure 5 compares the dose-response curve for our microblister data in the hairless guinea pig following HD-saturated vapor exposure with the available historical data base (19–22) of fluid-filled blisters observed in humans following a similar HD-vapor exposure technique. The correlation in this graph is apparent, under similar HD-vapor exposure conditions, both humans and hairless guinea pig appear to exhibit about the same incidence of epidermal-dermal separation.

Based on uniform response, ease of application, similarity of skin structure and pathology to humans, and ease of lesion assessment, the hairless guinea pig with saturated HD-vapor exposure to the skin is likely to be the animal model of choice for evaluating the effectiveness of candidate topical skin protectants, decontaminants, and treatment compounds for reducing HD-induced cutaneous lesions.

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